



IgG reactivity to phospholipid-bound β_2 -glycoprotein I is the main determinant of the fraction of lupus anticoagulant activity quenched by addition of hexagonal (II) phase phospholipid in patients with the clinical suspicion of antiphospholipid-antibody syndrome

OMID SAFA,^{*,^} LUCIANO CRIPPA,^{*} PATRIZIA DELLA VALLE,^{*} MARIA GRAZIA SABBADINI,[°] SILVANA VIGANÒ D'ANGELO,^{*} ARMANDO D'ANGELO^{*}

^{*}Coagulation Service and Thrombosis Research Unit and the [°] Department of Internal Medicine, IRCCS H.S. Raffaele, Milan, Italy

[^]Current address: Oklahoma Medical Research Foundation, 13th N.E. Street, Oklahoma City, OK, USA

ABSTRACT

Background and Objective. Autoantibodies to β_2 -glycoprotein I (β_2 -GPI) and/or prothrombin (FII) have been involved in the expression of lupus anticoagulant (LA) activity, an *in vitro* phenomenon associated with an increased risk of arterial and/or venous thromboembolic events. However, LA activity sustained by anti-FII antibodies has a much weaker association with thrombosis than LA activity sustained by anti- β_2 -GPI antibodies. Because assays aimed at detecting LA activity are now commercially available, we evaluated the relative sensitivity to anti-FII and anti- β_2 -GPI antibodies of a commercial LA assay in a consecutive series of patients with the clinical suspicion of anti-phospholipid antibody (APA) syndrome.

Design and Methods. One hundred and ten consecutive patients with the clinical suspicion of APA syndrome (primary in 39) and 36 healthy controls were evaluated for the presence of LA activity (LA, Sta-clot[®], Stago), anticardiolipin antibodies (Quanta Lite aCL IgG, IgM, Inova Diagnostics), and IgG binding to solid-phase and/or phospholipid (PL)-bound β_2 -GPI and FII by ELISA assays developed and optimized in our laboratory. Odds ratios for the association of IgG binding activity with LA and the aCL IgG status were calculated. In LA patients, dependency of LA potency (as assessed by clotting time prolongation in absence or presence of hexagonal phospholipid) on autoantibody titers was analyzed by the generalized linear model. Total IgG fractions were purified from selected patients to evaluate their ability to inhibit prothrombin activation at low FII concentration.

Results. Anticardiolipin antibodies (aCL) of the IgG or IgM type were found in 64 and 23 patients and LA activity in 49 patients. Anti- β_2 -GPI and anti-FII (solid-phase and PL-bound) IgG titers exceeding by more than 3 standard deviations the mean values observed in control subjects were found in 46 and 47 patients and in 56 and 30 patients respectively, with the highest titers detected in the subgroup of patients with

both LA and aCL IgG. The relative risk of LA for patients free of anti-FII and/or anti- β_2 -GPI IgG was 0.03 after stratification for the aCL IgG status. Anti- β_2 -GPI (solid-phase and PL-bound) IgG (RR 34.4 and 12.6) and anti-FII (solid-phase) IgG (RR 6.33) were all associated with LA activity. However, when taking into account co-existence of anti-FII and anti- β_2 -GPI IgG in the same patients, the relative risk of LA for patients with isolated anti-FII IgG (solid-phase and/or PL-bound) was 0.50, whereas it ranged from 4.24 to 8.70 for all the antibody combinations including anti- β_2 -GPI IgG. Anti- β_2 -GPI (PL-bound) and aCL IgG titers were the only significant predictors of LA potency determined in absence phospholipid (anti- β_2 -GPI IgG) or in presence of hexagonal phospholipid (aCL IgG). Total IgG fractions purified from 12 patients (6 with anti-FII IgG) did not significantly inhibit factor II activity up to a 150-fold molar excess.

Interpretation and Conclusions. These results highlight the high prevalence of anti-FII and anti- β_2 -GPI IgG in patients with the clinical suspicion of APA syndrome and particularly in the subgroup of patients with LA activity. The fraction of LA activity which can be quenched by addition of hexagonal phospholipid is, however, only dependent on IgG directed to PL-bound β_2 -GPI. Other antibodies associated with anticardiolipin IgG may explain residual clotting time prolongation observed in the presence of hexagonal phospholipid.

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Key words: anticardiolipin antibodies, lupus anticoagulants, anti- β_2 -glycoprotein I IgG, anti-prothrombin IgG, thrombosis, APA-syndrome

The mechanisms of action of lupus anticoagulants are ill understood. These anticoagulants were originally thought to be part of a family of circulating antibodies directed against anionic phospholipids¹ and it was held that their action in phospholipid-dependent clotting assays might be exerted through occupation by anticoagulant IgG or IgM of

Correspondence: Armando D'Angelo, M.D., Servizio di Coagulazione, Istituto Scientifico H.S. Raffaele, via Olgettina 60, 20132 Milan, Italy. Phone: international +39-02-26432228 - Fax: international +39-02-26432640 - E-mail: armando.dangelo@hsr.it

sites competing for the binding of procoagulant factors.^{2,3} It is now recognized that most antiphospholipid antibodies are directed to protein-phospholipid complexes.^{4,5} β_2 -glycoprotein I (β_2 -GPI;^{6,7}), prothrombin,^{8,9} protein S,¹⁰ protein C,^{10,11} thrombomodulin,¹² annexin V,¹³ high molecular weight kininogen,¹⁴ platelet activating factor,¹⁵ phospholipase A2¹⁶ and factor X⁴ have been identified as co-factors for the binding of these antibodies to phospholipid membranes. β_2 -GPI is the main co-factor for antibody binding to cardiolipin.⁷ Whether lupus anticoagulants all recognize protein co-factors for their expression is unknown. The majority of lupus anticoagulant (LA) antibodies are apparently β_2 -GPI-dependent,^{17,18} but prothrombin also has been invoked as a co-factor for the expression of LA activity.^{8,9,19,20} Because β_2 -GPI is an inhibitor of the intrinsic and extrinsic coagulation pathways,^{21,22} antibodies with LA activity may either increase β_2 -GPI binding to anionic phospholipid,^{23,24} impairing prothrombin conversion,²⁵ or recognize the phospholipid-prothrombin complex, inhibiting prothrombin⁷ and factor X⁸ activation. Recently, it was suggested that prothrombin-dependent and β_2 -GPI-dependent LA antibodies might be distinguished by their different activity in the dilute Russel viper venom time and the kaolin clotting time assay systems.²⁶ It is generally accepted that lupus anticoagulant activity is associated with an increased risk of venous and/or arterial thrombosis.^{27,28} However, the association of prothrombin-dependent LA antibodies with thrombotic manifestations appears much weaker than that of β_2 -GPI-dependent LA antibodies.^{26,29}

A series of commercial assays aimed at detecting LA activity has recently been introduced. Such assays are all based on the principle of correction of the abnormal clotting time prolongation with the addition of phospholipid, but they differ with respect to the phospholipid composition. Because lupus anticoagulant testing is mainly requested as an indicator of a potential thrombotic risk, knowledge of the relative sensitivity of commercial assays to prothrombin-dependent or β_2 -GPI-dependent LA antibodies is relevant to the evaluation of reagents in the clinical laboratory.

In this study, in a consecutive series of patients referred to our coagulation service with the suspicion of APA-syndrome, we evaluated the prevalence of anti- β_2 -GPI and anti-prothrombin IgG and their association with LA activity as detected by a commercial assay based on the use of hexagonal (II) phase phospholipid.

Design and Methods

Patients

One hundred and ten in-patients (72 women and 38 men, mean age at observation 38±15 years) referred to our laboratory from the Department of Internal

Medicine for evaluation of their anti-phospholipid antibody status and 36 healthy members of the laboratory staff (21 women and 15 men, mean age 41±9.8 years) were included in the study. Reasons for referral were clinical suspicion of autoimmune diseases (n = 82), recurrent abortions (n = 6), observation of isolated thrombocytopenia (<150,000/ μ L, n = 10), or unexplained APTT prolongation (n=12). Systemic lupus erythematosus (SLE) and other autoimmune diseases (thyroiditis, vasculitis, multiple sclerosis, myasthenia, and panarteritis nodosa) were diagnosed in 39 patients. The remaining patients had no well-defined autoimmune medical conditions. Objectively documented thrombotic episodes had occurred in 32 patients, arterial in 6, venous in 23, and both venous and arterial in 3. All patients with a history of thrombosis were studied at least one month after the occlusive event and they had no congenital thrombophilia defects;²⁸ sixteen patients were receiving oral anticoagulant treatment.

Laboratory methods

Blood sampling. All samples were collected in silicone vacutainer tubes containing 0.129 mol/L sodium citrate (Becton Dickinson, 1/10 blood volume) between 8.00 and 10.30 a.m. after overnight fasting. Platelet poor plasma was obtained by double centrifugation³⁰ at room temperature at 3,000 g for 15 min within 45 min of venesection. Lupus anticoagulant detection was carried out on fresh plasma. The remaining tests were performed on plasma aliquots (0.5 mL) snap-frozen with methanol and dry ice and stored at -80°C for no longer than 3 months. Pooled normal citrated plasma was obtained from 30 healthy subjects, aliquoted, snap-frozen and used within 30 days. Anticardiolipin antibody (aCL) titers were determined on serum samples.

Proteins. β_2 -glycoprotein I (β_2 -GPI) and prothrombin were purified from citrated outdated human plasma (3 liters) according to published procedures.^{31,32} The purity of protein preparations was more than 95% on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined spectrophotometrically from absorbance at 280 nm using extinction coefficients and molecular weights of 13.8 and 70,000 d for prothrombin,³² 9.4 and 50,000 d for β_2 -GPI.³³ After extensive dialysis against Tris-buffered saline, pH 7.5, concentrated protein aliquots (0.1 mL) were snap-frozen and stored at -80 °C until use.

Separation of total IgG fractions. Total IgG fractions were purified from selected patients and from normal pooled plasma obtained from 30 donors as previously described.³⁴ Citrated plasma aliquots (1 mL) were applied to a protein G column (Mab Trap GII, Pharmacia Biotech, Uppsala, Sweden). After washing the column, IgG were eluted in 3 mL of eluting buffer and brought to a pH of 7.5 with 1:10 volume of neutralizing buffer (all buffers supplied by the

manufacturer). The procedure was repeated several times, recovering from 10 to 45 mg of IgG for each patient. The IgG content of samples was determined by nephelometry (Behring, Marburg, Germany). After overnight dialysis in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA, USA) against 5 liters of 0.02 mol/L Tris-HCl, 0.1 mol/L NaCl (Sigma Chemicals, St. Louis, MO, USA) buffer, pH 7.5 (TBS), IgG were aliquoted, snap-frozen and stored at -80°C until use. In some cases, total IgG fractions were concentrated to 7 mg/mL (Amicon 30 Microconcentrator, W.R. Grace & Co., Danvers, MA, USA) prior to storage.

Lupus anticoagulant detection. Because 16 patients were on oral anticoagulation at observation, lupus anticoagulants (LA) were detected by observation of a prolonged clotting time with a commercial PTT reagent (PTT-LA[®], Stago, Gennevilliers, France) in a 50:50 mixture of test plasma with normal plasma. Plasmas with clotting times exceeding the ninety-fifth percentile of the distribution of a previously established control population underwent additional testing with the Staclot assays (Staclot-LA[®] Stago,³⁵). Confirmation of diagnosis was obtained by normalization or significant correction of the abnormality (i.e. shortening of the clotting time ≥ 8 sec) in the presence of hexagonal (II) phase phospholipid. All assays were carried out on citrated plasma after a double centrifugation procedure,³⁰ and results expressed as the ratio of the patient's clotting time divided by the clotting time of normal plasma (also centrifuged twice). In our hands, this diagnostic procedure has a sensitivity of 98% – at a set specificity of 95% – for the detection of LA by comparison with a panel of clotting assays – including the diluted Russell's Viper Venom time and the kaolin clotting time, and permits diagnosis in plasma from patients on oral anticoagulant treatment.³⁶

Detection of antibodies to cardiolipin (aCL) and of plasma IgG binding to solid-phase β_2 -GPI and prothrombin (FII). aCL IgG and IgM were tested by commercial ELISA methods on serum samples (Quanta Lite aCL IgG, IgM, Inova Diagnostics, San Diego, CA, USA) and results expressed as GPL or MPL units. Plasma IgG binding to solid phase β_2 -GPI and prothrombin (FII) were tested by a modification of the method described by Amiral *et al.*³⁷ Ninety-six well, γ -irradiated ELISA microtiter plates (Nunc-Immuno Plate Maxi Sorp. Roskilde, Denmark) were coated overnight with β_2 -GPI or prothrombin (10 $\mu\text{g}/\text{mL}$), 200 μL in TBS at room temperature. After blocking with 10% bovine serum albumin (BSA, Sigma) for one hour at room temperature, unbound material was removed by washing with 0.02 mol/L Tris-HCl, 0.2 mol/L NaCl, 0.1% Tween 20 (Pharmacia, washing buffer). All subsequent steps for the prothrombin ELISA were carried out in presence of 0.002 mol/L CaCl_2 . Test plasma (200 μL) diluted 1:50 in washing buffer containing 5% BSA was added to the wells and incubat-

ed under shaking (Dynatech Shaker Incubator, PBI, Milan, Italy) for one hour at room temperature, followed by repeated washings and by addition of a 1:10,000 dilution of peroxidase-conjugated rabbit anti-human IgG (DAKO, Glostrup, Denmark). After one hour, repeated washings removed unbound secondary antibody and the peroxidase substrate (2,2-azino-di-3-ethyl-benzathiazoline sulfonate, ATBS, Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) added to the wells. Color development was measured at 405 nm after 30 min (Titertek Multiskan MCC, Flow Laboratories, Milan, Italy). All determinations were performed in duplicate. The analytical performance of the ELISA was evaluated by repeated testing (4 times in each plate for 5 different plates) of plasma from two patients with optical densities < 0.3 and > 0.8 . Within- and between-assay imprecision (CV) were $< 6\%$ and $< 9\%$ for both assays.

Detection of plasma IgG binding to anionic phospholipid and to phospholipid-bound β_2 -GPI and prothrombin (FII). γ -irradiated ELISA plates were coated under a continuous nitrogen stream with 100 μL of a 20:80 phosphatidylserine: phosphatidylcholine (Sigma) mixture (10 $\mu\text{g}/\text{mL}$) in ethanol. For the detection of IgG binding to phospholipid (PL)-bound β_2 -GPI, 200 μL of β_2 -GPI (10 $\mu\text{g}/\text{mL}$ in TBS) were added to the wells and incubated overnight at room temperature. After blocking and washing as described in the previous section, test plasma (200 μL) diluted 1:50 in washing buffer containing 5% BSA was added to the wells and incubated under shaking for one hour at room temperature, followed by repeated washings and addition of peroxidase-conjugated rabbit anti-human IgG. After one hour, repeated washings removed unbound secondary antibody and the peroxidase substrate added to the wells. Color development was measured at 405 nm after 30 min. All determinations were performed in duplicate. The analytical performance of the ELISA was evaluated by repeated testing (4 times in each plate for 5 different plates) of plasma from two patients with optical densities < 0.25 and > 0.8 . Within- and between-assay imprecision (CV) were 4.6% and 7.4%. For the detection of IgG binding to PL-bound FII, PL-coated wells were blocked, followed by repeated washings and addition of 200 μL of TBS containing 5% BSA, 0.002 mol/L CaCl_2 , 0.1% Tween 20 and FII (10 $\mu\text{g}/\text{mL}$). All further steps reported above included the presence of 0.002 mol/L CaCl_2 in the buffers. All determinations were performed in duplicate. The analytical performance of the ELISA was evaluated by repeated testing (4 times in each plate for 5 different plates) of plasma from two patients with optical densities < 0.25 and > 0.5 . Within- and between-assay imprecision (CV) were 4.3% and 6.6%.

Binding of proteins to plastic or coated PL was monitored in a Vmax EASIA Reader (Medgenix, Belgium) by using polyclonal rabbit anti- β_2 -GPI or anti-prothrombin IgG (Figure 1). The apparent K_d for sol-

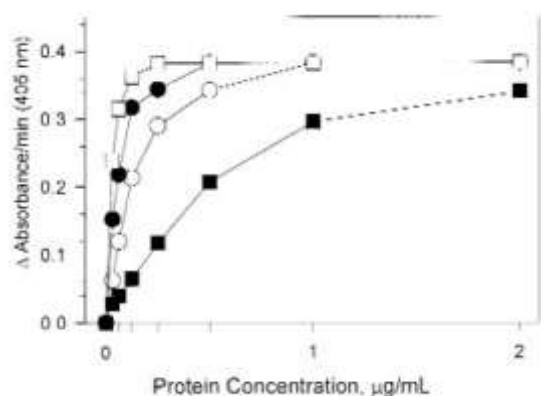


Figure 1. Assay optimization. Proteins at the indicated concentrations were coated onto the wells or incubated onto wells previously coated with the phospholipid mixture as described in the *Design and Methods* section. Binding of β_2 -glycoprotein I was detected by addition of peroxidase-conjugated polyclonal rabbit anti- β_2 -glycoprotein I IgG (1 $\mu\text{g}/\text{mL}$); binding of prothrombin was detected by addition of affinity-purified polyclonal rabbit anti-prothrombin IgG (1 $\mu\text{g}/\text{mL}$) followed by peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution). Changes in absorbance at 405 nm were recorded in a V_{max} reader. Open circles: solid-phase prothrombin; open squares: solid-phase β_2 -glycoprotein I; closed circles: phospholipid-bound prothrombin in the presence of 2 mM CaCl_2 ; open dotted circles: phospholipid-bound prothrombin in the presence of 2 mM EDTA; closed squares: phospholipid-bound β_2 -glycoprotein I.

id-phase and PL-bound proteins were: solid-phase β_2 -GPI: 4.5 nmol/L, PL-bound β_2 -GPI: 1.1 nmol/L, solid-phase FII: 0.3 nmol/L, PL-bound-FII: 4.0 nmol/L.

For the detection of plasma IgG binding to anionic phospholipid (PL), wells were coated, blocked, washed and incubated with test plasma as described above. Virtually identical OD was recorded both in the presence and absence of 0.002 mol/L CaCl_2 . The analytical performance of the ELISA was evaluated by repeated testing (4 times in each plate for 5 different plates) of plasma from two patients with optical densities < 0.22 and > 0.40. Within- and between-assay imprecision (CV) were 5.1% and 7.8%.

For all of the above described ELISAs, results – after subtraction of blank values – were considered abnormal when optical densities exceeded by more than 3 standard deviations (SD), the mean values observed in controls subjects.

Effect of IgG fractions on the prolongation of normal plasma's PTT-LA and on the activity of FII. The anticoagulant activity of IgG fractions purified from patients with lupus anticoagulants was tested in the PTT-LA assay. Normal pooled plasma (200 μL) was incubated with 50 μL of total IgG fractions (IgG concentrations ranging from 4.1 to 8.6 mg/mL) obtained from patients' plasma or normal plasma. Using the clotting time of the normal IgG-plasma mixture as the denominator, ratios > 1.30 were observed at patients' final IgG con-

centration ranging from 0.27 to 0.36 mg/mL.

Inhibition of FII activity by total IgG fractions from selected patients was tested by a commercial assay (STA Factor II, Stago) using a fully automated programmable coagulometer (STA, Stago). The software program was modified to avoid the initial 1:10 dilution of the test plasma. Normal plasma (25 μL) was added to 225 μL of diluent buffer containing increasing amounts of normal or patients' total IgG fraction (0-0.72 mg) and incubated for 1 hour at 37°C. Residual factor II activity in the presence of IgG fractions was extrapolated from the calibration curves obtained for normal plasma incubated with diluent buffer only. Controls were included in each analytical session using polyclonal rabbit anti-prothrombin IgG (0 to 0.25 mg/mL concentration in the incubation step).

Other laboratory methods. Polyclonal antibodies were raised in rabbits to β_2 -GPI by injection of 300 mg of the purified protein in Freund's complete adjuvant at multiple intradermal sites. Four weeks after the first immunization, 300 mg of the protein in incomplete adjuvant was injected at multiple subcutaneous sites. One week later, rabbits were bled and the serum tested by immunodiffusion. IgG were purified by protein G affinity chromatography followed by adsorption onto β_2 -GPI coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions (5 mg β_2 -GPI/mL of resin) and elution with 0.1 mol/L glycine-HCl, 0.5 mol/L NaCl, pH 2.7. After peroxidase-labeling (peroxidase from horse radish, Grad I, Boehringer Mannheim, Germany) using Nakane and Kawaoi's method,³⁸ the apparent Kd of the anti- β_2 -GPI polyclonal IgG for solid-phase β_2 -GPI was 22 nmol/L as tested by ELISA. Rabbit anti-prothrombin IgG was purified by protein G affinity chromatography from the commercial antiserum.

Statistical methods

For descriptive purposes, results are expressed as mean value \pm standard deviation (SD). Because normal distributions were not obtained by log-transformation of data, rank-transformation of data was adopted. One-way analysis of variance (ANOVA) and ANOVA for repeated measures were used to compare continuous variables among the groups of patients and the control population and the Spearman test was used to correlate autoantibody titers and clotting test aimed to evaluate LA potency. For multiple comparisons, p values were corrected according to Bonferroni's procedure. Odds ratios (OR) and Mantel-Haenszel relative risks (RR) were calculated by the χ^2 test. In LA+ patients, the generalized linear model (GLM) and the GLM for repeated measures were used to identify independent determinants of LA potency, as assessed by the PTT-LA 50:50 mixture assay and the two Staclot assays. Logistic regression analysis was used to evaluate the association of a previous history of thrombosis with

Table 1. Autoantibody status of the patient population (mean \pm SD) subdivided according to the lupus anticoagulant (LA) and anticardiolipin (aCL) IgG status.

	LA+/aCL+ (n = 31)	LA+/aCL- (n = 18)	LA-/aCL+ (n = 33)	LA-/aCL- (n = 28)	Controls (n = 36)	LA +/- <i>p</i>	aCL IgG +/- <i>p</i>
PTT-LA 50:50 mixture (ratio)	2.11 \pm 0.54 [#]	1.97 \pm 0.84 [#]	1.07 \pm 0.06	1.16 \pm 0.15	1.02 \pm 0.11	0.0001	ns
Staclot - hexagonal PL (ratio)	2.15 \pm 0.56 [#]	1.99 \pm 0.65 [#]	-	-	-	-	0.076
Staclot + hexagonal PL (ratio)	1.33 \pm 0.25	1.12 \pm 0.15	-	-	-	-	0.099
Hexagonal PL-dependent shortening of the clotting time (sec)	33.6 \pm 26.0	32.9 \pm 23.6	-	-	-	-	ns
aCL IgG (GPL units)	110.5 \pm 72.8 [#]	6.5 \pm 4.2 [#]	42.0 \pm 30.2 [#]	4.1 \pm 4.2 [#]	4.0 \pm 4.0	0.002	0.0001
aCL IgM (MPL units)	24.8 \pm 32.4 [#]	12.0 \pm 21.5 [#]	9.9 \pm 36.7 [#]	3.5 \pm 6.4 [#]	1.7 \pm 3.2	0.001	0.005
anti- β_2 -GPI (solid-phase) IgG (OD)	0.856 \pm 0.678 [#]	0.211 \pm 0.256 [#]	0.116 \pm 0.178	0.059 \pm 0.037	0.044 \pm 0.029	0.0001	0.003
anti- β_2 -GPI (PL-bound) IgG (OD)	0.695 \pm 0.694 [#]	0.072 \pm 0.053 [#]	0.081 \pm 0.155	0.035 \pm 0.026	0.022 \pm 0.015	0.0001	0.003
anti-FII (solid-phase) IgG (OD)	0.227 \pm 0.169 [#]	0.205 \pm 0.143 [#]	0.123 \pm 0.074 [#]	0.083 \pm 0.042	0.058 \pm 0.025	0.0001	0.005
anti-FII (PL-bound) IgG (OD)	0.102 \pm 0.182 [#]	0.078 \pm 0.080 [°]	0.073 \pm 0.055 [°]	0.068 \pm 0.086	0.030 \pm 0.020	ns	ns
anti-PL IgG (OD)	0.060 \pm 0.122 [*]	0.033 \pm 0.017	0.025 \pm 0.019	0.039 \pm 0.071	0.023 \pm 0.018	0.038	ns

Symbols indicate significance of differences vs. the control population: **p*<0.05; °*p*<0.01; #*p*<0.001. The independent associations of autoantibody titers with LA and aCL IgG status were evaluated by ANOVA restricting analysis to the patient population.

autoimmune diseases and the antibody titers. Differences were considered statistically significant with *p* values <0.05. The Systat® statistical software program was used for all calculations.

Results

aCL IgG and IgM were found in 18 patients; 46 had aCL IgG only and 5 patients aCL IgM only. PTT-LA 50:50 mixture ratios exceeding 1.25 were observed in 57 patients and in no control subject. With the Staclot assays, diagnosis of LA was established in 49 patients, associated with both aCL IgG and IgM in 15 patients, with aCL IgG only in 16 patients and with aCL IgM only in 3 patients. Patients with aCL IgG had a relative risk for LA activity of 1.09 (95% confidence interval, 0-1.80) after stratification for the aCL IgM status. Based on LA and/or aCL IgG results, the patient population was divided in 4 groups. Thirty-one patients had both LA and aCL IgG (LA+/aCL+); 18 patients had LA but tested negative for aCL IgG (LA+/aCL-); 33 patients had aCL IgG in their serum without detectable LA activity (LA-/aCL+), and 28 patients had neither LA nor aCL IgG (LA-/aCL-). The autoantibody status of the study population and its association with LA activity and aCL IgG are reported in Table 1. The potency of LA - as based on clotting time ratios - was similar in aCL IgG+ and aCL IgG- patients. With the exception of anti-FII (PL-bound) IgG, all autoantibody titers were higher in LA+ patients than in LA- patients, and with the exception of anti-PL and anti-FII (PL-bound) IgG titers, they were also higher in aCL IgG+ patients than in aCL IgG- patients (Table 1). As expected, contrary to anti- β_2 -GPI (solid-phase and PL-bound) IgG titers,

anti-FII (solid-phase), but not anti-FII (PL-bound) IgG titers were only weakly correlated to aCL IgG titers in the patient population (Figure 2). Anti-PL IgG titers were not correlated with aCL IgG titers (*r* = 0.039).

The numbers and percentages of patients with IgG binding to β_2 -GPI or FII (solid-phase or PL-bound) are reported in Table 2. IgG binding to anionic phospholipid was observed in 5 patients only (3 with and

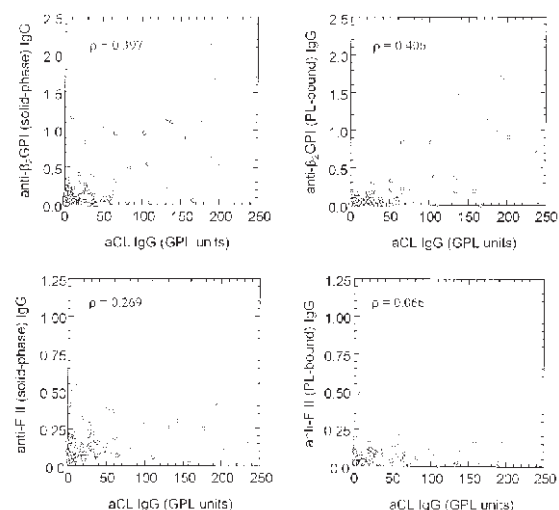


Figure 2. Correlations (Spearman *r* values) of anti- β_2 -glycoprotein I and anti-FII (solid-phase and phospholipid-bound) IgG titers (O.D.) with aCL IgG titers in the patient population. Correlations were statistically significant for anti- β_2 -glycoprotein I IgG (*p* <0.0001) and anti-FII (solid-phase) IgG titers (*p* = 0.022).

Table 2. Prevalence of IgG binding activity to β_2 -GPI and FII IgG (solid-phase and PL-bound) in the patient population. Odds ratios (OR) with 95% confidence intervals for LA activity and presence of aCL IgG and relative risk (RR) for LA activity stratified by aCL IgG status are shown.

	LA+/aCL+ (n = 31)	LA+/aCL- (n = 18)	LA-/aCL+ (n = 33)	LA-/aCL- (n = 28)	OR (95% CI *) for LA +	OR (95% CI) for aCL IgG +	Mantel-Haenszel RR (95% CI) for LA + stratified by aCL IgG +/-
Nihil	-	1 (5.6)	15 (45.5)	17 (60.7)	0.02 (0.005-0.14)	0.48	0.03 (0.01-0.11)
anti- β_2 GPI (solid-phase) IgG	28 (90.3)	10 (55.6)	7 (21.1)	1 (3.6)	22.9 (7.63-71.9)	3.84 (1.54-9.71)	34.4 (12.0-98.2)
anti- β_2 GPI (PL-bound) IgG	28 (90.3)	7 (38.9)	9 (27.3)	3 (10.7)	10.2 (3.88-27.5)	4.93 (1.94-12.8)	12.6 (4.86-32.4)
anti-FII (solid-phase) IgG	22 (71.0)	15 (94.4)	13 (39.4)	6 (21.4)	6.82 (2.71-17.5)	1.43	6.33 (2.75-14.6)
anti-FII (PL-bound) IgG	10 (32.3)	3 (16.7)	12 (36.4)	5 (14.3)	0.95	2.49	0.86

IgG binding activity was considered present with optical densities exceeding, by more than 3 standard deviations, the mean values observed in controls subjects.

*95% CI shown only for OR and/or RR significantly different from 1.

Table 3. Prevalence of IgG binding activity to β_2 -GPI and FII IgG (solid-phase and PL-bound, isolated or in combinations) in the patient population. Odds ratios (OR) with 95% confidence intervals for LA activity and presence of aCL IgG and relative risk (RR) for LA activity stratified by aCL IgG status are shown.

	LA+/aCL+ (n = 31)	LA+/aCL- (n = 18)	LA-/aCL+ (n = 33)	LA-/aCL- (n = 28)	OR (95% CI *) for LA +	OR (95% CI) for aCL IgG +	Mantel-Haenszel RR (95% CI) for LA + stratified for aCL IgG +/-
Anti- β_2 GPI (solid-phase) IgG	-	1 (5.6)	-	-	∞	0	2.03
Anti- β_2 GPI (PL-bound) IgG	1 (3.2)	-	-	3 (10.7)	0.40	0.23	0.56
Anti- β_2 GPI (solid-phase and PL-bound) IgG	7 (22.6)	1 (5.6)	-	-	$\infty^\#$	5.52	11.3 (1.46-87.9)
All isolated anti- β_2 GPI IgG	8 (25.8)	2 (11.1)	-	3 (10.7)	4.96 (1.15-20.6)	1.17	4.24 (1.04-17.3)
Anti-FII (solid-phase) IgG	1 (3.2)	5 (23.8)	4 (12.1)	2 (7.1)	1.28	0.47	1.34
Anti-FII (PL-bound) IgG	-	-	3 (9.1)	2 (7.1)	0	1.08	0
Anti-FII (solid-phase and PL-bound) IgG	1 (3.2)	-	2 (6.1)	3 (10.7)	0.23	0.70	0.36
All isolated anti-FII IgG	2 (6.4)	5 (23.8)	9 (27.3)	7 (25.0)	0.47	0.59	0.50
Anti-FII + anti- β_2 GPI (solid-phase) IgG	1 (3.2)	4 (22.2)	-	1 (3.6)	6.82	0.13	5.33
Anti-FII + anti- β_2 GPI (PL-bound) IgG	-	1 (5.6)	1 (3.0)	-	1.25	0.71	1.28
Anti-FII (solid-phase and PL-bound) + anti- β_2 GPI (PL-bound) IgG	-	1 (5.6)	1 (3.0)	-	1.25	0.71	1.28
Anti-FII (PL-bound) + anti- β_2 GPI (solid-phase and PL-bound) IgG	1 (3.2)	-	1 (3.0)	-	1.25	∞	1.21
Anti-FII (solid-phase) + anti- β_2 GPI (solid-phase and PL-bound) IgG	11 (35.5)	2 (11.1)	2 (6.1)	-	10.7 (2.09-44.3)	5.61 (1.11-27.5)	8.20 (2.00-33.5)
Anti-FII (solid-phase and PL-bound) + anti- β_2 GPI (solid-phase and PL-bound) IgG	8 (25.8)	2 (11.1)	4 (12.1)	-	3.65	5.08 (1.00-26.1)	3.00
All combinations of anti-FII and anti- β_2 GPI IgG	21 (67.7)	10 (55.6)	9 (27.3)	1 (3.6)	8.78 (3.31-23.9)	2.81 (1.13-7.08)	8.70 (3.55-21.3)

IgG binding activity was considered present with optical densities exceeding by more than 3 standard deviations the mean values observed in controls subjects.

*95% CI shown only for OR and/or RR significantly different from 1; $^\#p < 0.000001$.

2 without LA and aCL), and always in association with antibody species reacting to β_2 -GPI, FII or both. Anti- β_2 -GPI (solid phase and PL-bound) IgG were observed in 46 (41.8%) and in 47 (42.7%) patients.

Anti-FII (solid-phase and PL-bound) IgG were observed in 56 (50.9%) and in 30 (27.3%) patients. Table 2 also shows the association of the different IgG autoantibody species with LA activity and the

presence of aCL IgG, and the relative risk for LA activity contributed by the autoantibody species after stratification for the aCL IgG status. Only anti- β_2 -GPI IgG was significantly associated with an increased risk for the presence of aCL IgG. Taking such risk into account, the relative risk for LA activity contributed by anti- β_2 -GPI (solid-phase) IgG binding activity was greater than that contributed by anti- β_2 -GPI (PL-bound) IgG or anti-FII (solid-phase) IgG. IgG binding to PL-bound FII was not associated with LA activity. In contrast, patients with no IgG binding activity to either FII or β_2 -GPI had a markedly reduced risk of LA activity.

The data reported in Table 2 do not account for the effects of combinations of IgG autoantibody species, which are detailed in Table 3. Isolated anti- β_2 -GPI (solid-phase and/or PL-bound) IgG binding activity was observed less frequently than isolated anti-FII (solid-phase and/or PL-bound) IgG binding activity (11.8% vs. 20.9%), whereas co-existence of the two IgG antibody species was more frequently detected (37.3%). At variance with isolated anti- β_2 -GPI IgG, isolated anti-FII (solid-phase and/or PL-bound) IgG binding activity did not show significant association with LA activity and the aCL IgG⁺ status, whereas a relative risk for LA activity ranging from 4.24 to 8.70 was calculated for all combinations including anti- β_2 -GPI (solid-phase and/or PL-bound) IgG binding activity.

The above reported analysis of the influence of IgG autoantibodies on LA activity is insensitive to differences in autoantibody titers among patients with IgG binding activity to either β_2 -GPI, FII or both. Thus, we first evaluated in LA patients the correlation of autoantibody titers with the clotting tests aimed at

detecting LA activity and then used a generalized linear model to identify independent predictors of LA potency for each of the clotting tests. Table 4 shows that neither anti-FII (PL-bound) nor anti-PL IgG and aCL IgM titers were associated with LA potency in any of the three clotting tests. Only anti- β_2 -GPI (PL-bound) IgG titers showed a significant correlation with LA potency in the PTT-LA 50:50 mixture assay and in the Staclot assay carried out in presence of hexagonal phospholipid. In the latter assay only, LA potency showed a strong correlation with aCL IgG titers and a correlation of borderline significance with anti- β_2 -GPI (solid-phase) IgG titers.

In LA⁺ patients, anti- β_2 -GPI (solid-phase) and anti- β_2 -GPI (PL-bound) IgG titers showed a strong correlation ($r = 0.714$, $p < 0.0001$), and both autoantibody titers were also strongly correlated to the aCL IgG titers ($r = 0.580$ and 0.597 , $p < 0.0001$). Independent predictors of LA potency for each of the three clotting assays were thus analyzed in a generalized linear model, including the autoantibody titers among the independent variables. Only anti- β_2 -GPI (PL-bound) IgG titers were significant predictors of LA potency as evaluated by the assays carried out in the absence of hexagonal phospholipid (PTT-LA 50:50 mixture, $p = 0.006$; Staclot - hexagonal phospholipid, $p = 0.027$), accounting for 16.0% to 17.0% of the variation in LA potency. With the assay carried out in presence of hexagonal phospholipid, the aCL IgG titer was the only significant predictor ($p = 0.0001$) and accounted for 24.5% of the variations in Staclot ratios. The generalized linear model for repeated measures was also used to identify predictors of the changes in Staclot ratios resulting from the addition of hexagonal phospholipid. Only anti- β_2 -GPI (PL-bound) IgG titers were significantly associated to such changes ($p = 0.004$).

Because it has been reported that anti-FII autoantibodies are responsible for LA activity and they are so by interfering with the activation of factor II,²⁰ it cannot be ruled that our failure to observe any effect of anti-FII IgG on LA activity may have depended on the commercial assay adopted in this study to identify the presence of lupus anticoagulants. Thus, we investigated the inhibitory effect of anti-prothrombin IgG on factor II activity at low prothrombin concentration in a factor II assay carried out on normal plasma incubated with increasing concentrations of IgG fractions purified from normal pooled plasma from 4 patients free of LA (2 with and 2 without anti-FII IgG binding activity) and from 8 patients with LA (4 with and 4 without anti-FII IgG binding activity). Whereas total inhibition of factor II activity was attained at 0.2 mg/mL concentration of the polyclonal anti-prothrombin IgG fraction, no significant inhibition of factor II activity was obtained in either group of patients up to a 3.2 mg/mL total IgG concentration, representing a 150-fold molar excess of total IgG over the FII concentration in the assay (Figure 3).

Table 4. Correlations (ρ values and p values) of autoantibody titers with clotting time ratios in the 49 patients with LA activity.

	PTT-LA 50:50 mixture		Staclot (- hexagonal PL)		Staclot (+ hexagonal PL)	
	ρ	p	ρ	p	ρ	p
aCL IgG	0.142	ns	0.151	ns	0.473	0.005
aCL IgM	-0.026	ns	0.045	ns	-0.021	ns
Anti- β_2 -GPI (solid-phase) IgG	0.080	ns	0.038	ns	0.382	0.052
Anti- β_2 -GPI (PL-bound) IgG	0.401	0.044	0.259	ns	0.408	0.031
Anti-FII (solid-phase) IgG	0.059	ns	0.159	ns	0.115	ns
Anti-FII (PL-bound) IgG	0.030	ns	-0.268	ns	-0.099	ns
Anti-PL IgG	-0.092	ns	0.022	ns	0.049	ns

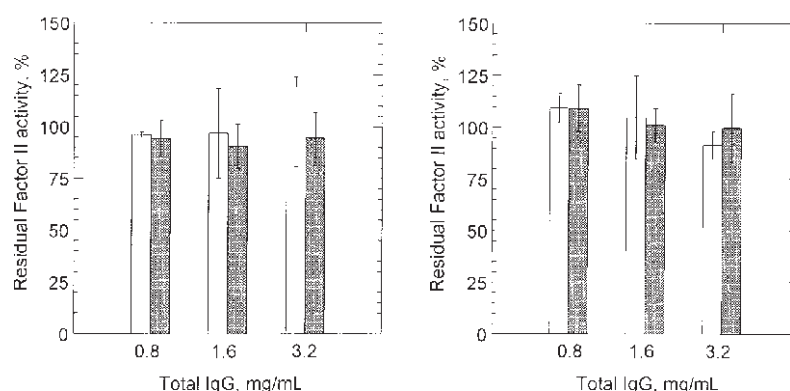


Figure 3. Inhibition of factor II activity by total IgG fractions. Total IgG fractions were purified from 12 patients and their effect on factor II activity evaluated as described in the *Design and Methods* section. Concentrations reported on the x axis refer to total IgG concentrations in the incubation step with plasma factor II. Left panel: residual factor II activity after incubation with total IgG fractions from patients free of lupus anticoagulant activity, with (closed bars, n = 2) or without (open bars, n = 2) anti-FII IgG. Right panel: residual factor II activity after incubation with total IgG fractions from patients with lupus anticoagulant activity, with (closed bars, n = 4) or without (open bars, n = 4) anti-FII IgG. Total inhibition of factor II activity was observed at 0.2 mg/mL concentration of the polyclonal rabbit anti-prothrombin IgG fraction.

The association of LA, aCI IgG, aCL IgM, anti- β_2 -GPI and anti-prothrombin IgG titers (solid-phase) with history of thrombosis in this series of patients was previously reported.²⁸ By including IgG titers to PL-bound β_2 -GPI and prothrombin in the logistic regression analysis, only the presence of LA was associated with a previous history of thrombosis in the 110 patients (OR = 4.3, 95% confidence interval: 1.98-9.29).

Discussion

This study was carried out with the aims of detecting the prevalence of anti- β_2 -GPI and anti-prothrombin IgG in a consecutive series of patients clinically suspected by having APA syndrome, primary or secondary, and of evaluating the association of such antibodies with the presence of lupus anticoagulant activity as detected by a commercial APTT-based assay.

In a series of 110 patients with the clinical suspicion of APA syndrome, we observed IgG directed to β_2 -GPI and FII (solid-phase and/or PL-bound) in 84% and 78% of 49 patients with lupus anticoagulant activity and in 17% and 43% of patients free of LA. Arvieux *et al.* in a series of 139 patients with LA found a prevalence of autoantibodies directed to β_2 -GPI and FII – of the G and M isotype – in 56% and 57% of patients.³⁹ Differences in the analytical systems adopted and in the criteria for diagnosis of lupus anticoagulant activity may at least partially explain the higher prevalences observed in our series of patients.

In spite of the high prevalence of both antibody species in patients with LA, the anti- β_2 -GPI, but not the anti-FII IgG titers were significantly associated with the potency of lupus anticoagulant activity. Lupus anticoagulant activity was detected in our

series of patients using a commercially available battery of tests, based on a screening aPTT assay carried out at a low phospholipid concentration, an additional aPTT assay carried out at a higher plasma dilution and a confirmatory test which includes hexagonal phospholipid in the assay mixture. Our results strongly suggest that all of the lupus anticoagulant activity inhibited by the addition of hexagonal phospholipid is closely dependent on anti- β_2 -GPI IgG, and especially on the antibody species reacting toward PL-bound β_2 -GPI. Heterogeneity of anti- β_2 -GPI antibodies has been reported recently by Arvieux *et al.*⁴⁰ It has been reported that prothrombin-dependent and β_2 -GPI-dependent LA antibodies can be distinguished by their different activity in the dilute Russell's viper venom time and the kaolin clotting time assay systems.²⁶ Because β_2 -GPI-dependent antibodies, in addition to inhibiting thrombin generation,^{17,25} also delay the inhibitory effect of β_2 -GPI on the generation of factor Xa by factor IXa and VIII,^{41,42} overall plasma assay systems such as kaolin clotting time are potentially less sensitive to the anticoagulant effects of such antibodies than the diluted Russell's viper venom time, which is independent of factor X activation. The reverse may be true for prothrombin-dependent LA antibodies which, in addition to inhibit the activation of human^{8,9} – and to a lesser but significant extent – bovine prothrombin,⁴³ also prevent factor X activation via recognition of the complex of lipid-bound prothrombin.⁹ Because we used an APTT-based assay system to detect the presence of lupus anticoagulant activity and observed dependency of LA potency on anti- β_2 -GPI IgG titers only, the observations of Galli *et al.*²⁶ probably cannot be applied to the general population of patients with lupus anticoagulants. Permpikul *et al.*⁹ used the dilute

Russell's viper venom time to detect lupus anticoagulant activity and observed dependence on prothrombin, but not on β_2 -GPI, of LA activity in all their patients' plasma samples. Using purified components, they found that whereas inhibition of prothrombin activation by lupus anticoagulant IgG was markedly enhanced upon preincubation of the antibodies with prothrombin, calcium ions and PS-PC vesicles, inhibition of the IXa/VIIIa/phospholipid catalyzed activation of factor X did not specifically require preincubation of IgG with prothrombin, calcium ions and PS-PC vesicles.⁹ When translated to overall plasma assay systems to detect lupus anticoagulant activity (APTT, kaolin clotting time), obviously lacking the preincubation step, such observations would be in line with the latter mechanism – i.e. inhibition of factor X activation – as being responsible for most of the detectable plasma LA activity.

There are two additional findings of this paper which deserve attention. Although incubation of purified IgG fractions with factor II did not show selective inhibition of factor II activity by anti-FII IgG, there was a trend to lower factor II activity levels in experiments in which incubation was carried out with total IgG fractions from patients with LA (Figure 3). Because factor II activity is measured in a prothrombin time assay, this observation may be related to our findings of the interference of lupus anticoagulant activity in prothrombin time assays aimed at monitoring oral anticoagulant treatment.⁴⁴ Interestingly, the residual clotting time prolongation observed in the presence of hexagonal phospholipid was no longer dependent on the anti- β_2 -GPI IgG titers, but was related to the aCL IgG titer. This suggests that in addition to β_2 -GPI-dependent LA IgG antibodies, other mechanisms may be responsible for LA activity in patients clinically suspected of having APA syndrome.

In conclusions, the principle of correction of plasma LA activity by the addition of hexagonal (II) phase phospholipid is sensitive to β_2 -GPI-dependent IgG. Because β_2 -GPI-dependent LA antibodies confer a significant thrombotic risk, at variance with prothrombin-dependent LA antibodies, commercial reagents containing this phospholipid mixture are suited for use in the clinical laboratory.

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OS and PDV designed and performed the experiments described in this paper. MGS and LC were responsible for patient referral to our laboratory. ADA and SVD wrote the manuscript. All authors were equally responsible for conception and design of the study and approved the final version of the paper. The order of authorship takes into account the time, work and scientific contributions of all authors.

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